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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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☐ *Additional inventors are being named on the ___ separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

Triacylglycerol-Deficient Fission Yeast and Its Use

Direct all correspondence to: **CORRESPONDENCE ADDRESS**

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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☐ Application Data Sheet. See 37 CFR 1.76

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Respectfully submitted,

SIGNATURE _____ Date 7/9/03

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TRIACYLGLYCEROL-DEFICIENT FISSION YEAST AND ITS USES

FIELD OF INVENTION

[0001] The invention relates to novel fission yeast that is triacylglycerol (TAG)-deficient. The novel yeast is susceptible to lipotoxicity and lipoapoptosis and is therefore useful in the study of lipotoxicity and lipoapoptosis and to identify compounds for the treatment or prevention of lipotoxicity and lipoapoptosis and disorders associated with lipotoxicity and/or lipoapoptosis, such as type II diabetes and cardiomyopathy. The novel yeast is also useful in the study of compounds involved in the synthesis of TAG and to identify inhibitors of those compounds, which inhibitors may be useful in the treatment or prevention of obesity.

BACKGROUND OF INVENTION

[0002] TAGs are important energy-storage molecules in most eukaryotes. Mice lacking partial ability to synthesize TAGs were resistant to diet induced obesity, most probably due to increased energy expenditure. Inhibitors of a mammalian diacylglycerol acyl-transferase gene DGAT1 and possibly other similar enzymes thus represent exciting novel drugs which might cure obesity (Chen and Farese, 2000). To date, few effective therapies are available for people suffering from obesity.

[0003] TAG and fatty acids overload in non-adipose tissues, e.g. pancreas, heart, muscle, could damage those tissues by a process called lipoapoptosis. Death of pancreatic beta cells is key to the pathogenesis of type II diabetes while death of cardiomyocytes could lead to heart failure. To date, our understanding of the molecular mechanisms underlying lipoapoptosis is limited and no therapies exists to prevent or slow down lipoapoptosis.

[0004] There is no simple yet powerful model system for developing anti-lipoapoptosis therapies to treat Type II diabetes and cardiomyopathy. A TAG-

deficient budding yeast strain and a knock-out mouse strain with reduced TAG in certain tissues exist but neither showed any phenotypic relevance to lipoapoptosis.

SUMMARY OF INVENTION

[0005] The invention provides a TAG-deficient fission yeast. The term TAG-deficient is used to describe a lack of detectable TAG synthesis that may result, for example from defective TAG synthesizing enzymes expressed at normal levels or a low or lack of expression of functional TAG synthesizing enzymes. In one embodiment, the yeast is *Schizosaccharomyces pombe Δplh1 Δdga1* double deletion mutant. The level of TAG synthesis may be detected by standard techniques. For example, cellular TAG levels may be detected by separating the cellular lipids by thin layer chromatography and then staining the separated lipids with iodine vapor.

[0006] The TAG-deficient fission yeast is susceptible to lipoapoptosis and lipotoxicity. Therefore, the yeast strains of the invention may be used to study the processes and molecules involved in the lipoapoptosis and lipotoxicity. TAG-deficient fission yeast represents the first unicellular model system that is suitable for the study of lipoapoptosis and lipotoxicity. Lipoapoptosis appears to be critical to the development of type II diabetes and cardiomyopathy. The yeast strains according to the invention may therefore be used to screen for compounds that inhibit lipoapoptosis and lipotoxicity and that may be therapeutically useful in disorders associated with lipoapoptosis or lipotoxicity, including but not limited to, type II diabetes and cardiomyopathy. The yeast strains may be advantageously used in high thruput screening of compounds.

[0007] Thus, the invention provides a method of screening or identifying compounds that inhibit or prevent lipoapoptosis or lipotoxicity, for example in pancreatic beta cells and cardiomyocytes, including compounds effective in the treatment or prevention of disorders associated with lipoapoptosis or lipotoxicity such as type II diabetes and cardiomyopathy. Such compounds may include small molecules and bioactive agents such as proteins, peptides, antibodies, hormones,

lipids and nucleic acids. In one embodiment, the method comprises the steps of growing a culture of fission yeast according to the invention, such as *Schizosaccharomyces pombe* Δ*plh1* Δ*dga1* double deletion mutant, treating the culture with a compound, exposing the treated culture to conditions that are suitable for inducing lipoapoptosis in an untreated culture and determining whether the treated culture has undergone lipoapoptosis.

[0008] The yeast strains according to the invention can also be used to study compounds involved in TAG synthesis and to identify inhibitors of those compounds and which inhibitors may be effective in treating or preventing disorders associated with TAG, such as obesity. Thus, the invention also provides a method of screening or identifying compounds that inhibit TAG synthesis, including compounds effective in the treatment of obesity. In one embodiment, the inhibitors of TAG synthesis may be identified by transforming TAG-deficient fission yeast such as *Schizosaccharomyces pombe* Δ*plh1* Δ*dga1* double deletion mutant with a mammalian gene encoding a protein involved in TAG synthesis, such as human DGAT, growing transformed yeast cell in culture, treating the culture with a compound and determining if any TAG is synthesized by the transformed yeast cell.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0009] We have created a yeast (*Schizosaccharomyces pombe*) strain, which is free of TAG, by deletion of two genes: *dga1* and *plh1*. Fission yeast is extremely easy to manipulate at molecular level when compared with most other experimental systems. To develop and analyze DGAT (an important mammalian TAG synthesizing enzyme) inhibitors, a system will be needed to produce, maintain and analyze the mammalian enzyme. The ideal system should have zero or extremely low endogenous activity and should be easy to manipulate at molecular level. The yeast strain of the invention can produce and harbor the mammalian enzyme whose DGAT activity can be easily assayed. Thus, our yeast strain may be used to study the structure and function of compounds involved in TAG synthesis, including mammalian DGATs, and to identify the active sites of these compounds. In one embodiment, the TAG-

deficient fission yeast further comprises exogenous mammalian gene such as a mammalian DGAT gene. In addition, the yeast can be used to identify or screen compounds effective in inhibiting compounds involved in TAG synthesis, including mammalian DGAT and which may therefore be effective in treating or preventing disorders associated with TAG, such as obesity.

[0010] Our yeast strain undergoes lipoapoptosis when challenged with fatty acids. Free fatty acids play a key role in the pathogenesis of type II diabetes and many studies suggested that high level of plasma free fatty acids and excessive accumulation of fatty acids in non-adipose tissues causes insulin resistance and cell death, especially apoptosis of the pancreatic beta cells. The *S. pombe* mutant cells deficient in TAG synthesis may serve as an excellent model system to study the molecular mechanisms of lipotoxicity and lipoapoptosis, since the effect of fatty acids on cell growth are more pronounced and can be easily detected in these mutants. Furthermore, the TAG-deficient *S. pombe* strains could offer a new platform to screen for compounds that might prevent fatty acids-induced lipoapoptosis, including compounds which may be effective in the treatment or prevention of disorders associated with lipotoxicity and lipoapoptosis, including type II diabetes and cardiomyopathy.

[0011] All documents referred to herein are fully incorporated by reference.

[0012] Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. All technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art of this invention, unless defined otherwise.

[0013] The word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to". The following examples are illustrative of various aspects of the invention, and do not limit the broad aspects of the invention as disclosed herein.

EXAMPLE 1

[0014] See attached manuscript.

Schizosaccharomyces pombe cells deficient in triacylglycerols synthesis undergo apoptosis upon entry into stationary phase

Summary

Triacylglycerols (TAG) are important energy storage molecules for nearly all eukaryotic organisms. In this study, we found that two gene products (Plh1p and Dga1p) are responsible for the terminal step of TAG synthesis in the fission yeast *Schizosaccharomyces pombe* through two different mechanisms: Plh1p is a phospholipid diacylglycerol acyltransferase (PDAT) while Dga1p is an acyl-CoA: diacylglycerol acyltransferase (DGAT). Cells with both *dga1*$^{+}$ and *plh1*$^{+}$ deleted (DKO cells) lost viability upon entry into stationary phase and demonstrated prominent apoptotic markers. Exponentially growing DKO cells also underwent dramatic apoptosis when briefly treated with diacylglycerols (DAG) or free fatty acids. We provide strong evidence suggesting that DAG, not sphingolipids, mediates fatty acids-induced lipoapoptosis in yeast. Lastly, we show that generation of reactive oxygen species (ROS) is essential to lipoapoptosis.

INTRODUCTION

Triacylglycerols (TAG) are important energy-storage molecules which can be found in almost all eukaryotes. In mammals, TAG synthesis plays essential roles in a number of physiological processes, including intestinal fat absorption, energy storage in muscle and adipose tissue and lactation. It also contributes to pathological conditions such as obesity and hypertriglyceridemia (1). TAG synthesis through both of the glycerol-3-phosphate pathway and the monoacylglycerol pathway is acyl-CoA dependent. The transfer of an acyl group from acyl-CoA to diacylglycerols (DAG) catalyzed by the enzyme diacylglycerol acyl-transferase (DGAT) is regarded as the only committed reaction in TAG synthesis in the glycerolipid pathway, since DAG is diverted from membrane glycerolipid biosynthesis (2). Two distinct mammalian DGAT genes have been identified recently. DGAT1 was cloned based on its sequence homology to genes involved in sterol esterification (3, 4). DGAT2 was identified by its homology to a DGAT isolated from the fungus *Mortierella rammaniana* (5, 6). Other acyl-CoA dependent TAG synthesizing enzymes are likely present but are yet to be identified. In addition, acyl-CoA independent TAG synthesis was also shown to exist in eukaryotes. A DAG transacylase, which synthesizes TAG from two DAGs, was purified from rat intestinal microsomes and its activity was comparable to that of DGAT (Lehner and Kuksis, 1993).

Four genes, i.e. *DGA1*, *LRO1*, *ARE1* and/or *ARE2*, have been found to encode proteins capable of synthesizing TAG in the budding yeast *Saccharomyces cerevisiae (S. cerevisiae)* (8, 9, 10, 11). Dga1p is highly homologous to mammalian DGAT2 while Lro1p encodes a protein with significant sequence similarity to the mammalian enzyme lethithin cholesterol acyltransferase (LCAT). Dga1p utilizes acyl-CoA to esterify DAG while Lro1p transfers an acyl group from a phospholipid molecule to the *sn*-3 position of DAG. Dga1p and Lro1p

mediate the bulk of TAG synthesis; however, in their absence, 2-4 % of normal TAG synthesis could still be detected. It was later determined that Are1p and Are2p, two acyl-CoA sterol acyltransferases in yeast, are responsible for this residual activity. When all four genes are deleted simultaneously, synthesis of both sterol esters and TAG is completely blocked. However, no obvious growth defects were detected in the budding yeast cells completely free of either TAG or neutral lipids. This is rather surprising because neutral lipids have long been regarded as a safe depot for polar and potentially toxic lipids such as fatty acids, DAG or sterols. A recent study has proven that synthesis of TAG prevents fatty acids-induced lipotoxicity in mammalian cells (12).

The fission yeast *Schizosaccharomyces pombe (S. pombe)*, similar to the budding yeast, is genetically tractable and equipped with a rich repertoire of molecular tools and a completely sequenced genome (13, 14). Although the fission and budding yeasts are as divergent from each other as each from the mammals, *S. pombe* has been shown to have greater similarity to mammals at least in certain steps of cell division and in aspects of stress signaling (15). The enzymes and pathways of lipid metabolism, their physiological significance, and their resemblance to mammalian systems are largely unexplored in *S. pombe*. In this work, we describe the identification of two genes: *plh1*$^+$ and *dga1*$^+$, which encode enzymes that are responsible for the bulk of TAG synthesis in the fission yeast. We provide convincing evidence that fission yeast cells defective in TAG synthesis undergo apoptosis upon entry into stationary phase. The important role of DAG in the induction of lipoapoptosis is also investigated.

Experimental procedures

Yeast strains, general techniques and reagents. Schizosaccharomyces pombe strains MBY257 (*h-, his3-D1, ade6-M210, leu1-32, ura4-D18*) and MBY266 (*h+, his3-D1, ade6-M210, leu1-32, ura4-D18*) were used in this study (16). Growth media (YES and EMM) and basic genetic, cell and biochemical techniques were used according to a previous report (17). Transformation of yeast was performed with electroporation, followed by prototrophic selection (18). Yeast extract, Yeast Nitrogen Base, Bacto-peptone and Bacto-agar were from Difco laboratories; D-dextrose, D-galactose and D-raffinose were from Sigma. 3, 3, 5, 5-Tetramethyi-1-1-pyrroline-n-oxide, 1,2-dioctanoyl-sn-glycerol, oleic acid, palmitic acid, 4', 6 diamidino-2-phenylindole (DAPI) and Nile Red were from Sigma. N-acetylsphigosine (C2-Cerimide) was from US Biological. [1-^{14}C] oleoyl-CoA, 1-Stearoyl-2-[^{14}C] arachidonyl-*sn*-glycerol, 1-palmitoyl-2 [1-^{14}C] oleoyl phosphatidylethanolamines (PE) and [9, 10(n) -^{3}H] oleic acid were from Amersham pharmacia biotech. In situ cell death detection kit and Annexin-v-fluos were from Roche.

Disruption of plh1$^+$, dga1$^+$ and pca1$^+$. For *plh1$^+$* gene disruption, the entire coding region of *plh1$^+$* was replaced by the *S. pombe his3$^+$* gene. Two pairs of primers: PLH1-55 (GGGGTA-CCACACCCTATTTGCAACA) and PLH1-53 (CCGCTCGAGGAATTGCTTGAGCAGC-AAC); PLH1-35 (CGGGATCCCGACAAACGAAT-ATGATAAA) and PLH1-33 (GCTCT-AGAGG-CTCCATAGAAGGTGAAG) were used to amplify DNA fragments flanking the coding region of *plh1$^+$*. The PCR products were cloned into a vector containing the *his3$^+$* gene to create a gene replacement cassette.

For *dga1$^+$* gene disruption, the entire coding region was replaced by the *S. pombe ura4$^+$* gene. Two pairs of primers: DGA1-55 (GGGGTACCGAATCCATGGGTAG-TGAT) and DG11-53 (CCGCTCGAGCCCGTTCTATATAAT CGT); DGA1-35 (C-GGGATCCCTTATTGG-

CCTATGCAATA) and DGA1-33 (GCTCTAGACTGAAT-GAATAT TAGTAACGC) were designed and a gene replacement cassette containing the *ura4*$^+$ gene was constructed to disrupt *dga1*$^+$.

For *pca1*$^+$ gene disruption, the entire coding region was replaced by the *kan*^{R} marker (19). Two pairs of primers: pca15 (ATAAGAATGCGGCCGCGGAAGAACTTTGACAC-GTT) and pca13 (GCTCTAGAGGAAGTTGGATAGTGCTT); pca25 (CCATCGATGTAGTTCC-ATCAGATATT) and pca23 (CCGCTCGAGGGTAGGTAGTATAGT-TAGA) were used to amplify DNA fragments flanking the coding region of *pca1*$^+$. The PCR products were cloned into pFA6akanMX4 (19), flanking *kan*^{R}.

Transformation of yeast: about 2 micrograms of gene replacement cassettes were used to transform wild type strains MBY266 and MBY257 by electroporation. Transformed cells were suspended in 200 µl 1.2M sorbitol and selected on EMM plates with appropriate amino acid supplements. Clones bearing the individual or double gene deletions were identified by diagnostic PCR with primers in the coding region of *ura4*$^+$ or *his3*$^+$ (GAGAAAGAATGC-TGAGTAG for *ura4*$^+$; and GAGTCT-TTAATTCATTAC for *his3*$^+$), and primers in region outside of the flanking fragment of *dga1*$^+$ or *plh1*$^+$ (CGATAGTAGTCAATACCAG and GTATATTAGTATTGCC-TAAT accordingly). The DKO strain was constructed by consecutive deletions of *plh1*$^+$ and *dga1*$^+$ in MBY266. The TKO strain was generated by deletion of *pca1*$^+$ from the DKO strain.

Expression plasmids construction. The entire open reading frame of *plh1*$^+$ was generated by RT-PCR using primers PLH5 (ACGCGTCGACCATGGCGT CTTCCCAAGAAGA) and PLH3 (TCCCCCGGGTTAATTTCTAGGTTTATCGAG) while the entire coding region of *dga1*$^+$ was amplified by PCR using the primers DGA1-5 (GGGAATTCCATATGTCAGA-

AGAAACATAA) and DGA1-3 (TCCCCCGGGTTAGGCTGACAACTTCAAT). The products were digested by *SmaI* and *SalI* and cloned into pREP41 or pREP42GFP, downstream of an *nmt1* promoter (20, 21). The open reading frame of DAG kinase was amplified from *E.coli* genomic DNA by PCR using the primers DGK5 (GGAATTCCATAT-GGCCAATAATACCACTG) and DGK3 (TCCCCCGGGTTATCCAAAATGCGACCAT) (22). The fragment was subcloned into the *SmaI* and *NdeI* sites of pREP41.

Cell viability assay. For cell viability at different growth phases, cells were grown to various densities in YES (determined by OD_{595}). The number of viable cells was obtained after cells were diluted properly in distilled water and plated in triplicates on YES agar. Colonies were scored after 3 days of incubation at 30°C. For cell viability after various treatments, cells were grown to early log phase (OD_{595}=0.1) before lipids or other chemicals were added. After treatment, cells were collected and viability was analyzed as described above.

DAG, fatty acids and ceramide treatment. For fatty acids treatment, palmitic acid and oleic acid were dissolved in chloroform as 500mM stock. Each microliter of fatty acids was dissovled in 12.5μl tyloxapol-enthanol (1:1) and added into growth medium. Wild type and DKO strains were grown to early log phase and then incubated in medium containing different concentrations (0.5mM, 0.8mM and 1mM) of palmitic acid or oleic acid for 0-3 hours. Control groups were cultured in the medium added with the same volume of tyloxapol-enthanol without fatty acids. After incubation, cells were analyzed for viability and DNA fragmentation. DiC8 DAG and ceramide were dissolved in DMSO. The working concentrations for DAG was 0.1mM, 0.2mM or 0.3mM while for ceramide was 10 μM or 20μM (23, 24).

Nile Red staining: Cells were grown to early stationary phase, washed with deionized H_2O two times and incubated with 1µg/ml of Nile Red (1mg/ml in acetone stock). Fluorescent images were obtained with a Leica DMLB microscope. (25)

Detection of apoptotic markers: All assays in this section were performed as previously described (26).

4', 6 diamidino-2-phenylindole (DAPI) staining: cells were fixed with 3.7% formaldehyde for 10 minutes, washed once with PBS containing 4% NP40 and twice with PBS, and then stained with DAPI. Cells were viewed using a Leica DMLB microscope.

Terminal deoxynucleotidyl transferase(TdT)-mediated dUTP nick-end labeling (TUNEL): Cells were fixed with 3.7% formaldehyde for 1 hr, digested with zymolase, washed with PBS, incubated in a permeabilization solution (0.1% Triton in 0.1% sodium citrate) for 2 minutes on ice, washed twice with PBS and incubated with 10 µl TUNEL mixtures for 1hour at 37°C. Cells were washed with PBS twice and were viewed using a Leica DMLB microscope.

Annexin V staining: Cells were washed in sorbitol buffer (1.2M sorbitol, 0.5mM $MgCl_2$, potassium phosphate, pH 6.8), digested with zymolase for 2hrs at room temperature, harvested, washed in binding buffer (10mM HEPES/ NaOH, 140mM NaCl, 2.5mM CaCl2, 1.2 M sorbitol), pelleted and resuspended in binding buffer. 2 µl annexin-FITC and 2 µl propidium iodide were added to38 µl cell suspension, and then incubated for 20 minnutes at room temperature. The cells were harvested, suspended in binding buffer, and applied to microscopic slides.

Production of reactive oxygen species (ROS) was detected by dihydroethidium (Sigma), which was used at 5μg per ml cell culture. After incubation for 10 minutes, cells were viewed under a Leica DMLB microscope through a Texas Red filter. The free radical spin trap reagent 3, 3, 5, 5,-tetramethyl-pyrroline *N*-oxide (TMPO) was used at 125μg per ml cell culture. Cells were pretreated with TMPO for 2 hours before lipids were added.

In vivo assay of oleate incorporation. The incorporation of [^{3}H] oleate into TAG was used as a measurement of DAG esterification essentially as described (9). Briefly, Cells were cultured in YES or EMM without appropriate nutrients for plasmid maintenance when necessary. Approximately 5 ml cells at logarithmic (log) phase (OD_{595} = 0.55-0.80) were pulsed with 5 μCi of [^{3}H]oleate at 30 °C for 30 minutes with shaking. Cells were washed twice with 0.5% tergitol, once with dH_2O, and lyophilized. The dried cell pellets were resuspended in 50 μl of lyticase stock solution (1700 units/ml in 10% glycerol, 0.02% sodium azide) and incubated at -70°C for one hour and at 30°C for 15 minutes. Lipids were extracted by hexane and analyzed by Thin Layer Chromatography (TLC). The plates were developed in hexane:diethyl ether:acetic acid (70:30:1) and stained with iodine vapor. Incorporation of label into lipids was determined after scintillation counting and normalization to a [^{14}C] cholesterol internal standard and cell dry weight. For each assay, minimums of three independent strains of each genotype were used. Statistical analysis was performed using paired t test.

Analysis of DAG accumulation by steady state labeling. Cells were grown for 18-25 hrs to mid-log phase or early stationary phase in media containing 1 μCi /ml [^{3}H]oleate (9). Cells were harvested and lipids were extracted, separated, visualized and quantified as described above.

Isolation of microsomes. Microsomes were isolated as described (27). Briefly, wild type and mutant strains were cultivated in 1 liter YES medium at 30°C overnight to log phase. Cells were collected through centrifugation. The pellets were washed with dH_2O, resuspended and incubated at 0.5g wet wt./ml in 0.1M Tris SO_4 (with 10mm-DTT) at room temperature for 10minutes. Cells were harvested, washed once with 1.2M sorbitol, and resuspended at 0.15g wet wt./ml in 1.2 M sorbitol (with 20mM K_3PO_4 and 0.5mg/ml lyticase), pH 7.2. Spheroplasts were formed after a 90-minute incubation at 30°C. Cells were washed twice with 1.2M sorbitol, resuspended and disrupted with 20 strokes in a Dounce homogenizer using a tight fitting pestle at 4°C. Homogenates were spun at 20,000g for 30 min. The pellets were discarded. The supernatants were collected and spun at 100,000g for 45 min. The final pellets containing microsomes were resuspended in 10mM-TrisCl pH 7.4. Protein concentrations were determined by a Bradford assay kit from Bio-Rad.

In vitro (microsomal) assay of DAG esterification. Enzyme activity was determined by the incorporation of [1-^{14}C] oleoyl-CoA, 1-stearoyl-2-[^{14}C] arachidonyl-sn-glycerol and 1-palmitoyl-2 [1-^{14}C] oleoyl phosphatidylethanolamines (PE) into TAG as described (9). Each standard assay was performed in triplicates in 150mM Tris-HCl pH 7.8 and final volume was 200 µl, containing 80 µg microsomal proteins, 15 µM BSA, 150 µM DAG, 8 mM $MgCl_2$,150 µM phosphatidylserine (PS)/phosphatidylethanolamines (PE) liposomes (1:1 molar ratio), and 50 µM oleoyl-CoA. All the assays were conducted at room temperature for 15min. For Plh1 activity assay, oleoyl-CoA was omitted while [^{14}C] PE was added in liposomes. Reactions were stopped by the addition of 6ml chloroform/methanol (2:1). Phase separation was induced by the addition of 1.2ml water. 1µl of [^{3}H] cholesterol and 15µg triolein were added as an internal standard and carrier, respectively. The lipid-containing phase was dried

with nitrogen and the lipids were dissolved in 100 µl choloroform for spotting on TLC plates. The plates were developed in hexane:diethyl ether:acetic acid (70:30:1) and TAG was quantified by scintillation counting.

Diacylglycerol kinase assay. The assay was conducted as described in the Biotrak assay reagents system (Amersham Biosciences). Wild type and DKO yeast cells were grown in YES medium to mid log phase and then treated with medium containing 0.8mM palmitate or oleate. Cells were collected at different time points (0, 30, 60 and 120 minutes). DAG was extracted with other lipids and quantified through a phosphorylation reaction catalyzed by a bacterial DAG kinase.

RESULTS

Identification of plh1+ and dga1+ in S. pombe. We searched the fission yeast genome database for homologous sequences to human DGAT1 (hDGAT1), human DGAT2 (hDGAT2) and the budding yeast *LRO1* using tBLASTX. A sequence with significant homology to hDGAT2 (40 percent identity at protein level) was identified and named *dga1*$^+$ (GeneDB systematic name: SPCC1235.15). In addition, as previously reported, an open reading frame highly homologous (45 percent identity at protein level) to the budding yeast *LRO1* was found in the fission yeast genome and named *plh1*$^+$ (for Pombe LRO1 Homolog 1, GeneDB systematic name: SPBC776.14) in this study. A few open reading frames showing limited homology to DGAT1 were also found but they are unlikely to play a role in TAG synthesis as suggested by a previous report (9). *plh1*$^+$ predicts a protein of 623 amino acids, with a putative transmembrane domain near its N terminus. Plh1p also has a conserved serine lipase motif HS(M/L)G between amino acids 292-296. *dga1*$^+$ encodes a 349-residue protein with at least one transmembrane domain. The region of the putative glycerol phospholipid domain in hDGAT2 was also found to be conserved in Dga1p (45% percent identity over 80 amino acids).

Deletion of plh1$^+$ *and dga1*$^+$ *resulted in a viable yeast cell without detectable TAG.* To determine whether Plh1p and Dga1p are involved in TAG synthesis in the fission yeast, we generated *Δplh1*, *Δdga1* single and *Δplh1 Δdga1* double deletion (referred to as the DKO strain thereafter) mutants by homologous recombination. All mutants were viable at 16°C, 30°C and 37°C on rich or minimal media and on different carbon sources (data not shown). We were also unable to observe any obvious morphological changes in the DKO cells under light microscope. To investigate whether cellular TAG mass was affected in these strains, cells were grown to mid-log phase and lipids

were extracted, separated by thin layer chromatography (TLC) and stained by iodine vapor. While the TAG mass in each single deletion mutant was visually indistinguishable from that of the wild type cells, virtually no TAG mass could be seen for DKO cells (data not shown). The sterol ester mass was clearly visible for all mutants, ruling out a lipid extraction error for the DKO strain. To further examine the ability of these strains to synthesize TAG, cells in log phase were pulse labeled with [^{3}H] oleate and its incorporation into TAG was measured (Fig.1A). No significant differences in oleate incorporation into TAG were detected between wild type and the *Δdga1* mutant. However, TAG synthesis was decreased by nearly 50% due to the loss of Plh1p. Most notably, the double mutant was almost totally deficient in TAG synthesis. In contrast, sterol ester biosynthesis was normal in all mutants (data not shown). In the budding yeast, it has been confirmed that Are1p and/or Are2p were responsible for the residual TAG synthesis activity (about three percent of wild type level). In *S. pombe*, there are two proteins (GeneDB systematic names: SPAC13G7.05 and SPCP1E11.05) which share strong homology with Are1p and Are2p. We have determined that these two homologs catalyze sterol esterification in *S. pombe* (data not shown); however, whether these proteins have a role in TAG synthesis remains to be examined. To confirm that either Plh1p or Dga1p was sufficient for TAG synthesis, we overexpressed *plh1*$^+$ and *dga1*$^+$ in wild type and DKO strains. Both genes were placed under the control of a modified *nmt1* promoter (21) and each gene was able to complement the TAG synthesis defect in the DKO mutant, indicating an overlapping function of these two genes (Fig. 1B). Overexpression of *plh*$^+$ and *dga1*$^+$ also caused a significant increase in TAG synthesis in WT and mutant strains, suggesting these genes could be regulated at transcription level. These results imply that TAG synthesis is mediated by two gene products in fission yeast while Plh1p

plays a major role at log phase. To further confirm the absence of TAG in the DKO strain, we treated yeast cells with Nile Red, a fluorescent dye with strong and specific affinity for neutral lipids (28). In both of the wild type and DKO cells, cytoplasmic fluorescent droplets could be seen in early stationary phase cultures. However, the number and intensity of the droplets observed in DKO cells was significantly less than those in wild type strains (Fig. 1C).

In vitro microsomal assays of DAG esterification. The results described above demonstrated the essential roles of Plh1p and Dga1p in TAG synthesis; however, they did not reveal the exact molecular function of these two proteins. Based on sequence homology and experimental data from previous studies (8, 9, 10, 11), it is highly likely that both of Plh1p and Dga1p carry out DAG esterification, with Dga1p functioning as an acyl-CoA DAG acyltransferase (DGAT) while Plh1p functioning as a phospholipid DAG acyltransferase (PDAT). To confirm this hypothesis, we analyzed DAG esterification using microsomes prepared from the wild type and deletion mutant strains. When [^{14}C]oleoyl-CoA and unlabeled diacylglycerol were added to the microsomes, the DGAT activity in Δ*plh1* cells was twice the level in WT cells while it was nearly undetectable in microsomes from *Δdga1* and DKO cells. When [^{14}C]diacylglycerol and cold acyl-CoA were used, no DAG incorporation into TAG could be detected in the DKO strain. The *Δplh* microsomes synthesized about 50% TAG of the normal microsomes while the *Δdga1* microsomes synthesized nearly the same amount of TAG as the wild type microsomes (Fig.2B). When 1-palmitoyl-2 [1-^{14}C] oleoyl phosphatidylethanolamines (PE) and unlabeled DAG were added, microsomes from wild type cells and *Δdga1* cells incorporated radiolabeled fatty acid into TG at similar rates (Fig.2C). This activity was absent in Δ*plh1* microsomes and in

microsomes prepared from the DKO strain, indicating that Plh1p mediates esterification of DAG using the *sn*-2 acyl group of PE as the acyl donor. Lastly, normal microsomes showed no incorporation of fatty acid from PE into sterol esters (data not shown), indicating that ergosterol is not a substrate for Plh1p under these assay conditions.

Cells deleted for both plh1$^+$ and dga1$^+$ underwent apoptosis upon entering stationary phase. Schaffer and colleagues have recently demonstrated that TAG synthesis protected against fatty acid-induced lipotoxicity in Chinese hamster ovary (CHO) cells (12). Other recent studies also implicated a critical role for TAG synthesis in cell viability in *Drosophila* and in the oleaginous yeast (29, 30). Previous reports indicated that the sole growth phenotype in budding yeast cells without neutral lipids was a prolonged lag phase with no significant change of growth in exponential or stationary phases. We examined the growth properties of wild type and DKO *S. pombe* cells and found that when stationary phase cells with the same OD_{595} value were used to start a growth culture, there was indeed a significant delay in the onset of log phase for DKO cells; however, when log-phase cells were used to start the culture, there was no such lag phase (data not shown). These data suggest that, among other possibilities, most of the double mutant cells in the stationary phase could not be revived. To test this hypothesis, a colony forming assay was performed (Fig. 3A) and as expected, most of DKO cells started losing viability upon entry into stationary phase. In addition, when the stationary-phase cells were stained with DAPI, remarkable nuclear DNA fragmentation was detected in the majority of the DKO cells, while no such fragmentation was observed in any WT cells (Fig. 3B). The mechanisms of this stress-induced cell death were further investigated by the TUNEL assay. Cleavage of

DNA during apoptosis produces free 3'OH termini, which can be effectively labeled by fluorescently tagged nucleotides in a process catalyzed by terminal deoxynucleotidyl transferase (TdT). It was found that at early stationary phase, DNA fragmentation occurs in the majority (over 50%) of mutant cells, as seen by the extensive TUNEL reaction (Fig. 3C). This extensive labeling was not observed in any of the wild-type cells. Log-phase cells in general did not show positive labelling, a result that is consistent with the findings that in the logarithmic growth phase, the double mutant has a comparable viability with the wild-type strain. The TUNEL positive phenotype suggested to us that the DKO cells could undergo apoptotic cell death when starved; therefore, we look for more apoptotic markers. In mammalian and *S. cerevisiae* cells, exposure of phosphatidylserine (PS) at the outer leaflet of the plasma membrane is an early marker for apoptosis (31). To test whether the same process occurs in *S. pombe*, spheroplasts of WT and DKO cells derived from stationary phase cultures were incubated with FITC-labeled annexin V. As shown in figure 3D, strong fluorescence could be seen in the periphery of about 10% of mutant cells, suggesting that phosphotidylserine is indeed exposed to the outer leaflet of plasma membrane. Membrane integrity was intact as propidium iodine (PI) was excluded from most of the annexin V positive cells. In contrast, no FITC fluorescence was observed for WT cells. Production of reactive oxygen species (ROS) represents another prominent marker for apoptosis in yeast (26). We treated WT and DKO cells with dihydroethidium, which can be oxidized by ROS to fluorescent ethidium. As shown in figure 3E, over 50% of mutant cells at stationary phase fluoresced strongly, whereas WT cells showed little or no fluorescence (Fig. 3E), neither did exponentially growing cells (not shown).

DAG accumulates in DKO cells and triggers apoptosis. Loss of viability, nuclear DNA fragmentation, exposure of PS and generation of reactive oxygen species lend strong support to the conclusion that these DKO cells underwent apoptosis upon nutrient starvation. Apoptosis has been shown to exist in the unicellular organism *S. cerevisiae*, with ROS playing a central regulatory role and the newly identified caspase homolog Yca1p as a possible central executioner (32, 33). However, how internal and environmental cues stimulate the production of ROS is largely unknown and whether there are other factors or pathways which might function independently of ROS or caspase to regulate apoptosis in yeast remains to be explored. We sought to understand the mechanism of this novel form of apoptotic cell death caused by the inability of DKO cells to produce TAG. *S. cerevisiae* cells begin accumulating neutral lipids upon exiting logarithmic growth phase, probably as a result of phospholipids remodeling by altering the activities of phosphatidate phosphotases (34). Due to the loss of DAG esterification capability in DKO cells, DAG and long chain fatty acids, two signaling molecules and major substrates for TAG synthesis, could accumulate upon entry into stationary phase and induce apoptosis. We therefore examined the quantity of DAG, palmitic and oleic acids, in WT and DKO strains. Steady state labeling experiments showed that there was almost 300% more DAG in DKO cells than in WT cells at early stationary phase (Fig. 4A), whereas there was little difference between the two strains at log phase. Surprisingly, no significant differences in free fatty acid levels between WT and DKO cells were observed as measured by GC-MS (not shown). This result suggests that accumulation of DAG might be the key to the death of DKO cells at stationary phase. The free fatty acids might have been incorporated into such molecules as DAG, ceramide, phospholipids and sterol esters etc. We reasoned that if the apoptotic cell death of the double mutant

cells was indeed caused by accumulation of DAG upon entry into stationary phase, we should be able to kill exponentially growing cells with exogenously added DAG. We therefore treated log-phase cells with a membrane permeable DAG analog called diC8 DAG (1, 2-dioctanoyl-*sn*-glycerol). Early log phase cells were treated with various concentrations of DAG and for 0-3 hours. As expected, prominent nuclear DNA fragmentation and cell death were observed in the DKO strain, but not the wild type. The percentage of cells showing DNA fragmentation generally increases with time (up to 3 hours) and concentration of DAG (up to 0.3mM). The results of cells treated with 0.2mM DAG for 2 hours are shown in figure 4 (4B, 4C, 4D).

Fatty acids induce apoptosis through DAG. To further investigate the possible role of DAG in yeast apoptosis, we focused our attention on free fatty acids. We reasoned that if DAG is indeed the apoptosis-inducing molecule in the DKO cells, treating the cells with free fatty acids would also trigger apoptosis because excessive free fatty acids would increase the production of, among other molecules, DAG. We treated WT and DKO cells with palmitate and oleate and not surprisingly, both of them caused the DKO cells to undergo apoptosis (Fig. 5, A, B and C). To prove that the fatty acids-induced apoptosis is caused by DAG, we first measured cellular DAG level after fatty acids were added to growth media. Since steady state labeling would not be a feasible method to estimate DAG in this case, a DAG kinase kit was used instead to estimate cellular DAG after cells were incubated with 0.8M palmitate or oleate for 2 hours. As expected, the DAG level in DKO cells increased from 1nmol/mg dry weight before addition of palmitate to 3.5 nmol/mg dry weight after a 2-hour incubation. In wild type cells, the change was mild (from 1 to 1.5 nM/mg dry weight). With oleate, there was a less but significant increase in DAG level (from 1 to

2.5 nM/mg dry weight) in DKO cells. The different effects of palmitate or oleate could be due to substrate preference of glycerol-3 phosphate acyltransferases in *S. pombe*. In fact, it has been demonstrated in *S. cerevisiae* that 16-carbon fatty acids are preferred substrates of Gat2p, one of the two newly identified enzymes that control the initial steps of glycerolipid synthesis (35). To further prove that fatty acids cause apoptosis through increased *de novo* synthesis of DAG, we tested whether removal of DAG could attenuate or reverse palmitate/oleate-induced apoptosis. A bacterial DAG kinase (DGK) was expressed in the double deletion strain under the control of a modified *nmt1* promoter (21, 22). The expression resulted in a DAG kinase activity of 35 pmol/min/mg as determined by ATP (γ-^{32}P) incorporation into phosphatidic acid (PA), three fold higher than basal activity. As shown in figure 6A, nearly 60 percent of cells survived as a result of DGK expression while only a 5% survival rate was observed in cells containing the empty plasmid. Further evidence was obtained by DAPI staining and the TUNEL assay. Significantly fewer cells with DGK expression showed DNA fragmentation and positive TUNEL reaction (Fig. 6B, 6C). Based on these results, we could conclude that the apoptosis inducing effect of fatty acids is mediated, if not exclusively, by DAG.

Palmitate can also induce *de novo* synthesis of sphingolipids, some of which are potent pro-apoptotic molecules in other experimental systems. To determine whether dihydrosphingosine (DHS), phytosphingosine (PHS), or ceramide plays a role in paltimate-induced apoptosis, WT and DKO cells were treated with various concentrations of DHS, PHS or C2-ceramide for different periods of time (0-3 hours). These compounds could kill *S. pombe* cells at high concentrations; however, no DNA fragmentation was observed (only the effect of a two-hour treatment by 20μM C2

ceramide was shown in Fig. 7A, 7B, 7C). These data are consistent with previous findings that ceramide cause cell cycle growth arrest, not apoptosis, in *S. cerevisiae* (24). We also examined the effect of fumonisin B1 (inhibitor of ceramide synthase) and myriocin (inhibitor of serine palmitoyl transferase) on the growth of double deletion cells treated with 0.8mM of palmitate. As expected, no rescue of apoptosis was observed and as a matter of fact, more cells underwent apoptosis in the presence of fumonisin B1 or myriocin (data not shown). These results suggest that sphingolipids are not involved in palmitate induced apoptosis in *S. pombe* and when sphingolipid synthesis is blocked, palmitate could be channeled to other pathways, such as the glycerolipid pathway.

Generation of ROS is essential to lipoapoptosis while deletion of a caspase homolog has no effect. Oxidative stress has been shown to act as a key regulator of apoptosis in *S. cerevisiae* (26) and in other organisms (36, 37). In addition, palmitate-induced lipoapoptosis in CHO cells required increased production of ROS (38). We therefore sought to determine whether generation of ROS is required for the death of DKO cells. We have shown that ROS were generated when DKO cells entered stationary phase (Fig. 3E). Incubation of log phase cells with DAG (not shown), oleate (not shown) or palmitate (Fig. 8C) also induced dramatic ROS production. To determine whether ROS are required for lipoapoptosis in our yeast strain, we examined the effect of the free radical spin trap TMPO on cell viability and DNA fragmentation. Mutant cells were pretreated with TMPO for 3 hours before 0.8 mM palmitate was added for 1 hour. As shown in figure 8, TMPO could effectively scavenge ROS (Fig. 8C) and prevent DNA fragmentation (Fig. 8B) and cell death (Fig. 8A).

The recent discovery of Yca1p, a yeast caspase homolog in *S. cerevisiae*, has generated much interest and excitement about regulators of apoptosis in yeast. We identified a Yca1 homolog in *S. pombe* (GeneDB systematic name: SPCC1840.04) and named it *pca1*$^{+}$, for pombe caspase 1. A triple deletion strain (*Δdga1 Δplh Δpca1*, referred to as the TKO strain thereafter) was created and surprisingly, no difference in the degree of cell death and DNA fragmentation was observed between the DKO and TKO strains when cells were grown to stationary phase or when log phase cells were treated with DAG or fatty acids (data not shown). Adding caspase inhibitor zVAD-fmk also failed to prevent cell death and DNA fragmentation in our experimental systems (data not shown). These results suggest that the Pca1p or caspase does not play an essential role in lipoapoptosis in the fission yeast.

DISCUSSION

In the current study, we identified two enzymes, Plh1p and Dga1p, which mediate the last step of TAG synthesis in the fission yeast *S. pombe*. Deletion of *plh1*$^+$ and *dga1*$^+$ caused cells to undergo apoptosis upon entry into stationary phase. We demonstrated, for the first time, that apoptotic cell death could result from deletion of endogenous genes in *S. pombe*. In addition, clear evidence was provided to show that DAG, not sphingolipids, induced lipoapoptosis in *S. pombe*. Elevation of cellular DAG might represent one of the most primitive proapoptotic signals. We further demonstrated that ROS is generated and required for lipoapoptosis in the fission yeast.

Similar to their *S. cerevisiae* counterparts, Dga1p has DGAT activity while Plh1p is a PDAT. One important difference is that unlike *S. cerevisiae*, whereas a small but significant amount of TAG can be detected in *LRO1* and *DGA1* double deletion cells, we were not able to observe any significant TAG synthesis when both *plh1*$^+$ and *dga1*$^+$ were deleted. This could be due to the sensitivity of our assay; however, it is more likely that the two yeasts are different in this respect. The requirement of two mechanistically different reactions to synthesize the same product would be meaningful to a cell if the two enzymes are differentially regulated, alternatively localized or preferentially recognized by different DAG species. In agreement with Oelkers et al. (10), our results indicate that Plh1p or the PDAT activity is responsible for the majority of TAG synthesis when yeast cells are undergoing exponential growth. Whether the DGAT activity is more important at stationary phase remains to be investigated. In addition, PDAT activity utilizes phospholipids, an essential component of all eukaryotic membranes. It is therefore conceivable that in addition to its role in TAG synthesis, Plh1p might function to modulate membrane lipid

composition and related cellular events such as cross membrane transport and vesicular trafficking. For instance, the yeast Sec14 pathway clearly demonstrated the dynamic interface between phospholipids metabolism and Golgi function (23). Lastly, although both DGAT and PDAT activities could be detected in the microsomal fractions in the budding yeast, the *S. pombe* Dga1p appears to localize exclusively to the lipid droplets while PDAT to the ER (data not shown). The significance of this pattern of localization remains to be understood.

The most important finding in this study is that *S. pombe* cells without detectable TAG undergo apoptosis upon entry into stationary phase. Apoptosis is a form of cell death that plays a critical role in the development and homeostasis of all multicellular organisms. Recent studies have proven that apoptosis also exists in unicellular organisms, such as the yeast *S. cerevisiae* (33). Various stimuli can cause apoptotic cell death in *S. cerevisiae*, including oxygen radicals, sexual pheromone, UV, salt stress and expression of proapoptotic mammalian genes, e.g. Bax (reviewed in 33). Mutations in certain *S. cerevisiae* genes could also trigger apoptosis (31; 39). All cases of apoptosis documented so far in *S. cerevisiae* were shown to be associated with increased production of ROS, which is believed to be a key regulator for apoptosis in both uni-and multi- cellular organisms. A recent landmark study identified a caspase related protease that appeared to regulate apoptosis in *S. cerevisiae*, further supporting a common mechanism underlying yeast and mammalian apoptotic processes (32). Apoptosis in *S. pombe* is much less well characterized and the only reported cases were induced by overexpression of mammalian Bak (40) or C. elegans *Ced-4* (41). In this report, we found that *S. pombe* cells unable to synthesize TAG lost viability upon entry into stationary phase and displayed prominent apoptotic

markers, which were not observed when cells were killed by treatment of C2 ceramide or sphingoid bases. One surprising result was the lack of effect on the death of DKO cells when the caspase homolog *pca1*$^{+}$ was deleted. In other experimental systems, caspase independent cell death pathways do exist and one of such pathways is controlled by AIF, the apoptosis inducing factor (42). There is a homolog to AIF encoded by the fission yeast genome and whether it is important to the death of DKO cells is under investigation. Interestingly, the Yca1p homolog in *Aspergillus nidulans* was not required for its apoptotic cell death induced by sphingoid long-chain bases (43). The molecular details of primitive apoptotic pathways in unicellular organisms therefore require further investigation. Nonetheless, our results added *S. pombe* to a growing family of unicellular organisms in which apoptotic cell death can be endogenically triggered.

The fact that DKO cells lost viability upon entry into stationary phase is both intriguing and informative. Normal yeast cells arrest cell growth and enter a resting state called stationary phase upon nutritional limitation (44). It is known that yeast cells accumulate neutral lipids after diauxic shift, possibly as a result of phospholipids remodeling. Although TAG might be required for yeast cells to survive stationary phase, possibly as an energy source, it is more likely that TAG serves as an inert storage depot for such bioactive molecules as DAG and fatty acids. Failure to convert DAG and fatty acids into TAG could result in deleterious consequences. In fact, Schaffer and colleagues have recently reported that accumulation of triglycerides protects against fatty acid induced lipotoxicity in mammalian cells (12). In addition, a mutation in *Drosophila* DGAT gene led to apoptotic cell death of egg chamber cells, although the exact mechanism was unclear (29). In the current study, we provided

several lines of convincing evidence supporting a critical role of DAG in the death of DKO cells: first, mutant cells grown in rich media accumulated DAG upon entry into stationary phase; second, exogenous diC8 DAG caused exponentially growing mutant cells to undergo apoptosis; third, addition of palmitate and oleate induced DAG synthesis and triggered apoptosis, which could be largely rescued by overexpression of a bacterial DAG kinase; forth, DHS, PHS or ceramide could kill mutant cells in a manner other than apoptosis. The mechanism by which DAG induces apoptosis is largely unclear. Protein kinases Cs (PKC) are a classical family of proteins that could be activated by DAG and the activated isoforms of PKC could be either pro-apoptotic or anti-apoptotic (45). PKC homologs do exist in *S. pombe*; however, its interaction with DAG and its role in apoptosis are yet to be established (46). It is highly likely that DAG might activate proapoptotic proteins other than PKC. For instance, a mammalian protein Munc13 could bind DAG and induce apoptosis when overexpressed (47). The next obvious challenge is to identify the target of DAG and how it activates the apoptotic pathway in the fission yeast. Lastly, although we have thus far focused on the role of lipids in causing apoptotic cell death, it is of importance to note that nutrient depletion not only changes cellular lipid metabolism, it is itself a common form of stress. In both yeast and mammalian cells, stress-activated MAP kinase pathways are known to play an important role in the activation of apoptosis (48, 49). It would be interesting to examine how the TAG deficient cells respond to other forms of stress, such as oxidative or osmotic stresses. The eventual apoptotic cell death reported here could probably result from the interplay between accumulation of toxic lipids (DAG) and stress signaling.

The fact that *S. cerevisiae* cells deficient in TAG or neutral lipid synthesis showed no obvious growth defects is puzzling. Sandager *et al.* reported a 3.7 fold decrease of DAG in cells deficient in neutral lipid synthesis at stationary phase in *S. cerevisiae* (8), which clearly is not the case in *S. pombe* mutants. This discrepancy highlights the differences between the two yeasts and emphasizes the necessity to conduct parallel studies in both model systems. *S. pombe* in this regard bears more resemblance to the situation in higher eukaryotes as recent data suggested that TAG synthesis could be an essential process for higher cells. *S. pombe* has been extensively used to study the molecular mechanisms of many aspects of cell physiology, including cell cycle and stress signaling; however, little work has been done in the area of lipid cell biology. Therefore, examination of certain aspects of lipid metabolism in the fission yeast might yield highly valuable information.

Free fatty acids play a key role in the pathogenesis of type II diabetes and many studies suggested that high level of plasma free fatty acids and excessive accumulation of fatty acids in non-adipose tissues causes insulin resistance and cell death, especially apoptosis of the pancreatic beta cells (50, 51). Using Zucker diabetic fatty *fa/fa* (ZDF) rats, Unger and colleagues showed convincingly that fatty acids and over-accumulation of TAG caused pancreatic beta cells to undergo lipoapoptosis, which was probably mediated by increased production of ceramide and nitric oxide (NO) (reviewed in 51). Studies by Schaffer and colleagues showed that ROS, rather than ceramide, was critical in the fatty acids- induced apoptosis of CHO cells (38). The involvement of DAG and protein kinase C (PKC) in palmitate-induced generation of ROS was demonstrated when cultured aortic smooth muscle cells were incubated with high level of palmitate (52). The *S. pombe* mutant cells deficient in

TAG synthesis may serve as an excellent model system to study the molecular mechanisms of lipotoxicity and lipoapoptosis, since the effect of fatty acids on cell growth are more pronounced and can be easily detected in these mutants. As shown in this study, we provided convincing evidence that DAG and ROS are critical for fatty acids-induced apoptosis in yeast. Powered by the genetic tractability of *S. pombe*, more components of the lipoapoptotic pathway are expected to be identified and characterized; and whether the same components and mechanisms exist in mammalian systems, especially during the course of the development of human type II diabetes, would be highly interesting and worthy of future investigation. Lastly, the TAG-less *S. pombe* strain could offer a novel platform to screen for compounds that might prevent fatty acids-induced lipoapoptosis.

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Figure Legends

Figure 1. Analysis of TAG synthesis *in vivo*. A. [^{3}H] oleate pulse labeling of wild type (WT) and deletion strains. WT, single and double deletion strains were grown to log phase and labeled with [^{3}H] oleate as described under "Experimental Procedures". DKO represents the *Δplh1Δdga1* double deletion strain. B. Overexpression of *plh1* and *dga1* in WT and DKO strains. WT and DKO strains were transformed with a vector control (pREP41), *plh1* and *dga1*. Cells were grown to log phase and pulse labeled with [^{3}H] oleate as described in 1A. *plh1*$^+$ and *dga1*$^+$ were expressed under the control of a modified *nmt1* promoter. C. Nile Red staining of WT and DKO cells. Cells were grown to early stationary phase and stained with 10mg/ml Nile Red.

Figure 2. In vitro assays of DAG esterification. Microsomes were extracted from WT and deletion strains grown to log phase. DAG esterification activities were assayed *in vitro* at 23°C for 15min with varying substrates. A, 50 μM [1-^{14}C] oleoyl-CoA and 150 mM 1, 2-dioleoyl-*sn*-glycerol. B, 200 μM 1-stearoyl-2-[1^{14}C] arachidonyl-*sn*-glycerol and 50mM oleoyl-CoA. *C*, 75 μM 1-palmitoyl-2 [1-^{14}C] oleoyl phosphatidylethanolamines and 150 μM 1, 2-dioleoyl-*sn*-glycerol. Assays were performed in triplicates. The amount of radioactivity incorporated into TAG in each assay using WT microsomes was set as 100%. Incorporation using microsomes from deletion strains were expressed as percentage of WT.

Figure 3. Double deletion (DKO) cells undergo apoptosis upon entry into stationary phase. A. Cell viability. At indicated cell densities, viability of WT and DKO stains was determined by counting the number of colonies after 1ml of each culture was plated; B. DAPI staining. WT and DKO cells were grown to early

stationary phase (OD_{595}=2) and stained with DAPI; C. TUNEL assay. WT and DKO strains were grown to early stationary phase and stained with TUNEL; D. Phosphatidylserine exposition. Early stationary phase cells were stained with FITC labeled annexin V and propidium iodine (PI); E. Generation of ROS. WT and DKO cells were grown to early stationary phase and incubated with dihydroethidium for 10 minutes. Fluorescence and phase contrast micrographs are shown. DIC: differential interference contrast microscopy.

Figure 4. DAG induces apoptosis at log phase. A. DAG quantification at log phase and early stationary phase. WT and DKO cells were labeled with [^{3}H] acetate to steady state for 18 hours (mid-log phase) or 25 hours (early stationary phase). Lipids were separated on TLC and the fraction corresponding to DAG was counted; B. Cell viability assay. WT and DKO strains were grown to early log phase (OD_{595}=0.1) and treated with 0.2mM diC8 DAG for 2 hours. Cell viability was determined by counting the number of colonies after 1ml of each culture was plated. DAPI staining (C) and TUNEL assay (D) were performed as in figure 3 except that cells were grown to early log phase and treated with diC8 DAG for two hours.

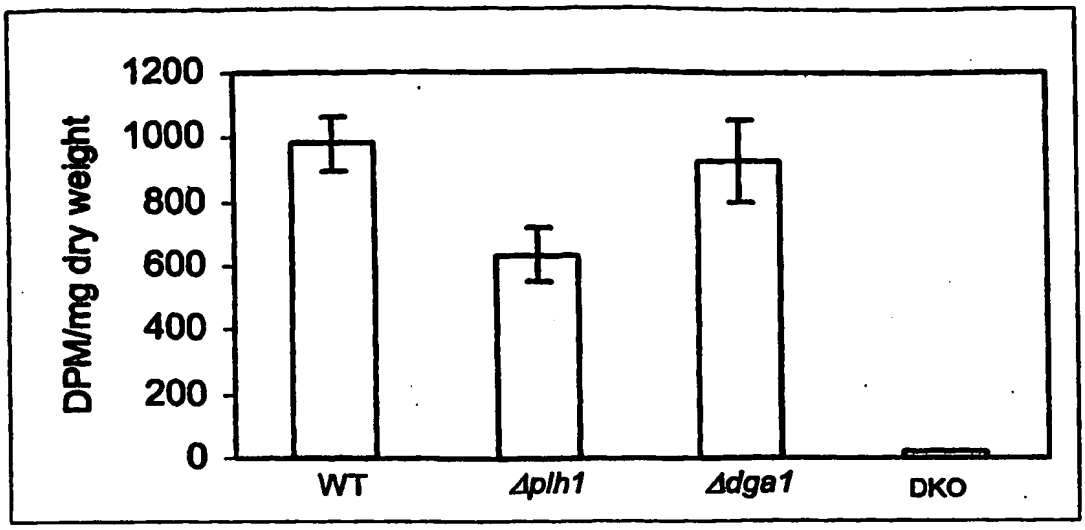
Figure 5. Palmitate or oleate causes exponentially growing cells to undergo apoptosis. Cells were grown to early log phase and treated with various concentrations of oleate or palmitate for two hours. Cell viability (A), DAPI (B) and TUNEL (C) assays were performed as described in figure 4. Only one concentration (1mM) of fatty acids was shown for DAPI and TUNEL assays.

Figure 6. Rescue of free fatty acids-induced apoptosis by DAG kinase expression. DKO cells with a control plasmid (*pREP41*) and with a plasmid expressing DAG kinase (*pREP41dgk*) were grown to early log phase and treated with 1mM palmitate or oleate for two hours. Cell viability, DAPI and TUNEL assays were performed as described in figure 4. Only the results of palmitate treatment are shown for DAPI and TUNEL.

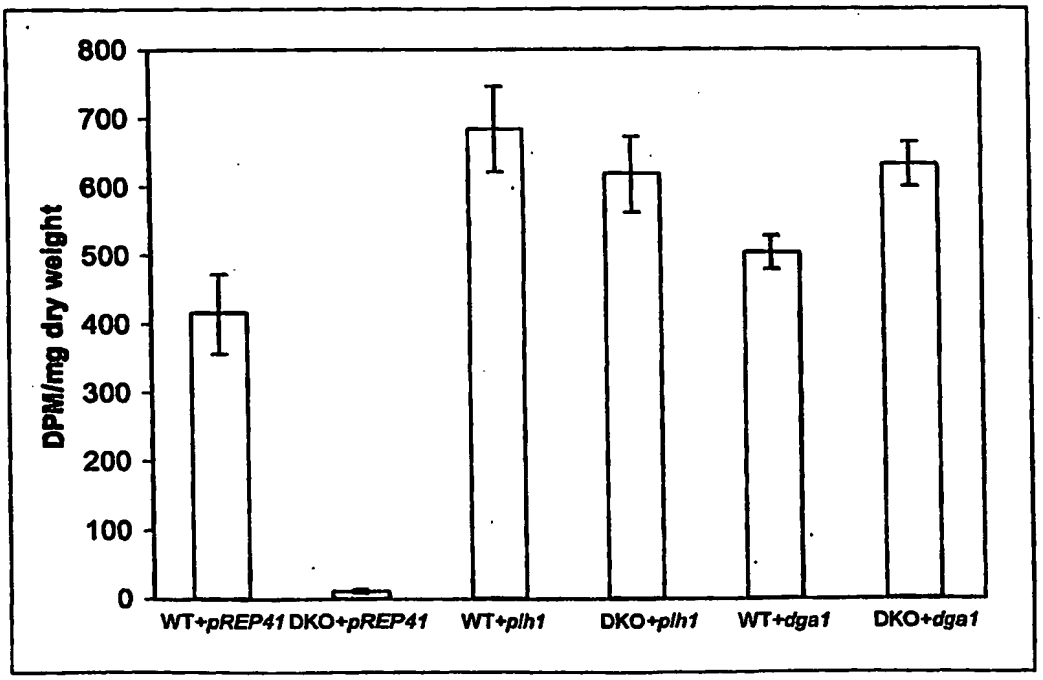
Figure 7. Sphingolipids are not involved in free fatty acids-induced cell death. DKO cells were grown to early log phase and treated with various concentrations of C2 ceramide for two hours. Cell viability (A), DAPI (B) and TUNEL (C) assays were performed as in figure 4. Only results of selected concentrations of C2 ceramide were shown for each assay.

Figure 8. Generation of ROS is essential to lipoapoptosis. Log phase WT and DKO cells were pretreated with or without 125μg/ml of TMPO for two hours and then incubated with 1mM palmitate for two more hours. Colony forming (A), DAPI staining (B) and ROS production (C) were performed as described in figure 4 and "experimental procedures".

A

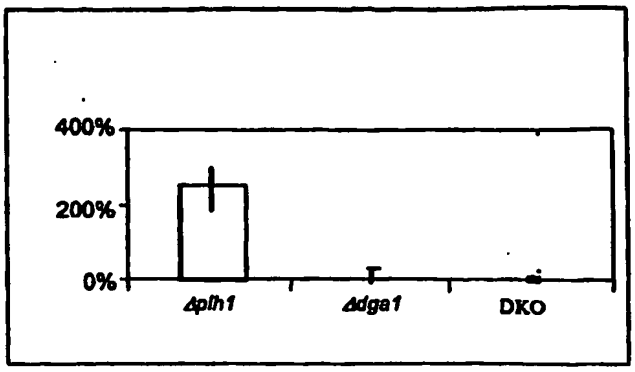


B



Zhang et al., Figure 1, A and B

A



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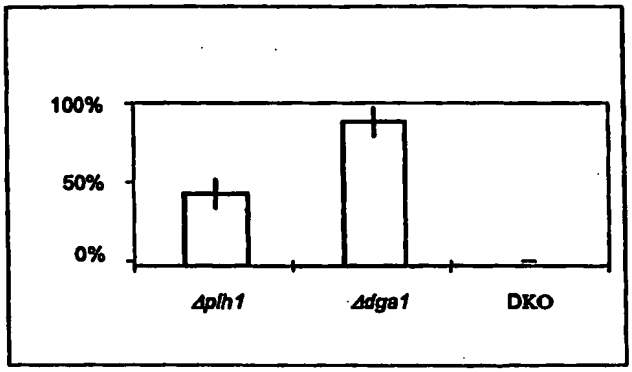
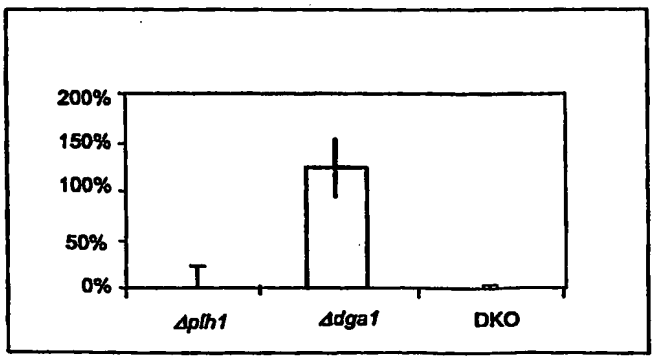
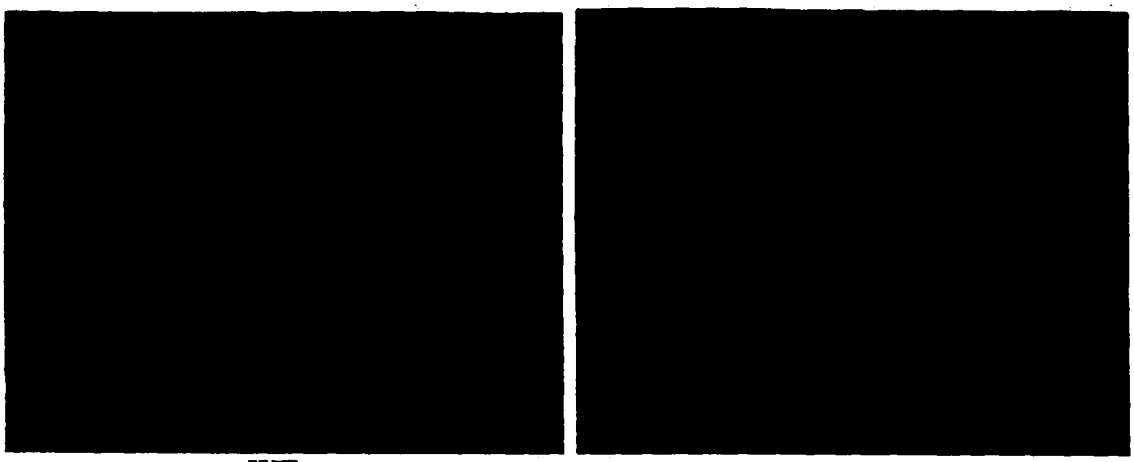


C



Zhang et al., Figure 2 A, B, C.

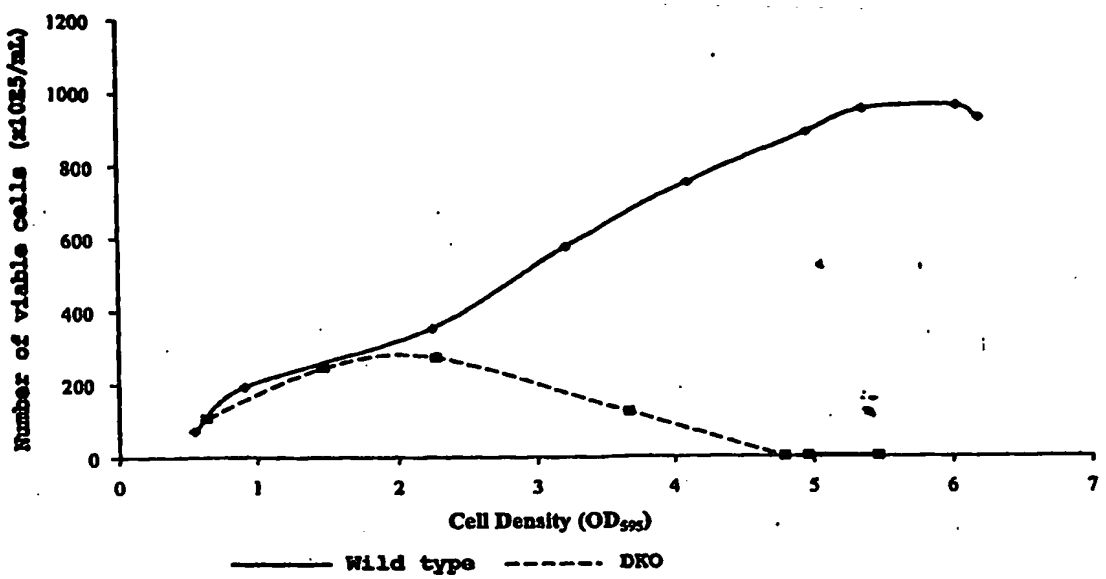
C



WT DKO

Zhang et al., Figure 1 C

A



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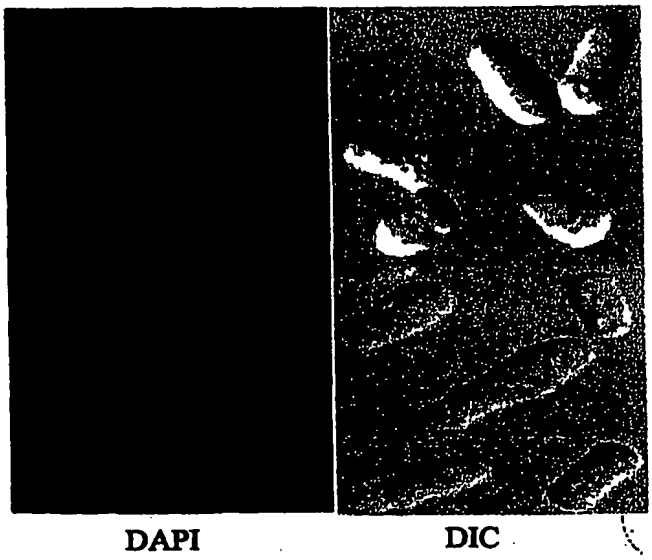
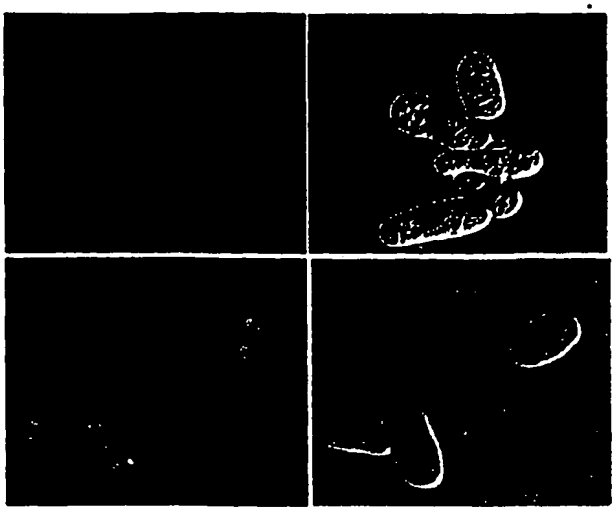
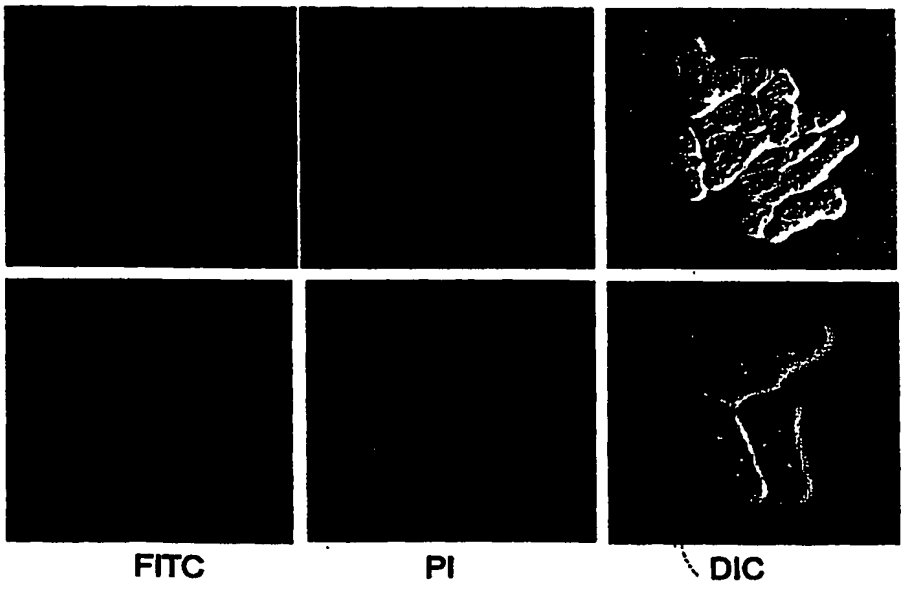


Zhang *et al.*, Figure 3 A, B

C

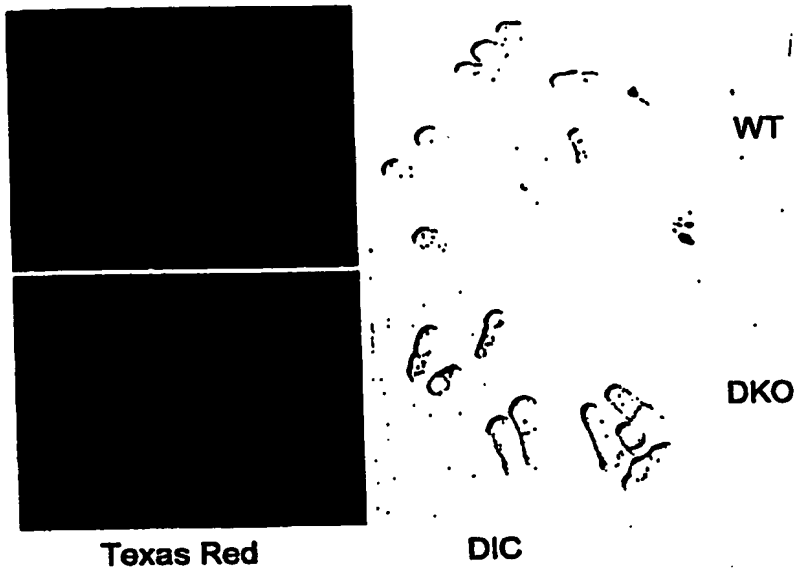


D



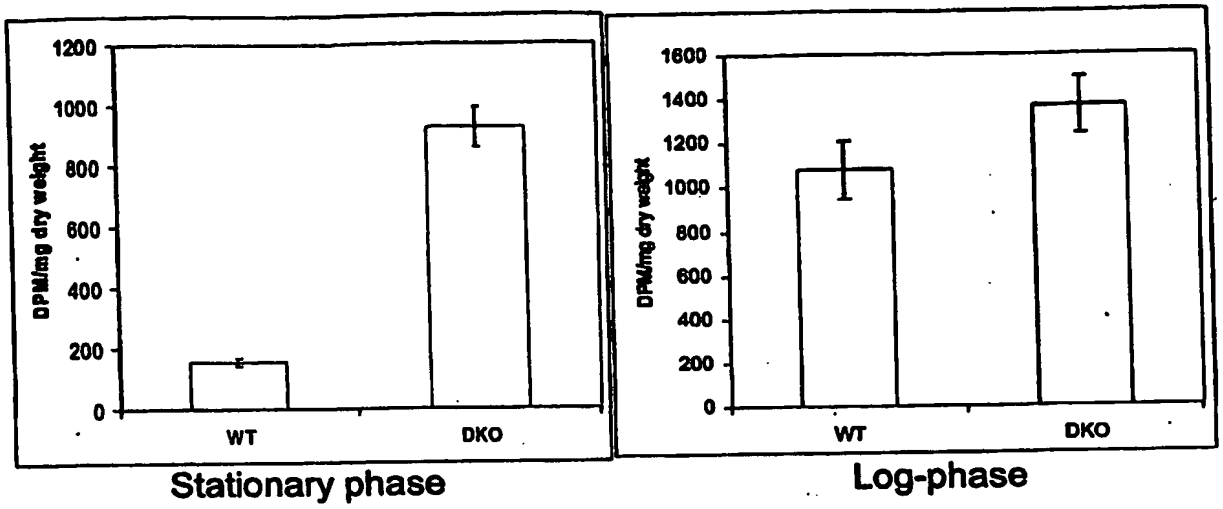
Zhang *et al.*, Figure 3 C, D

E



Zhang *et al.*, Figure 3 E

A



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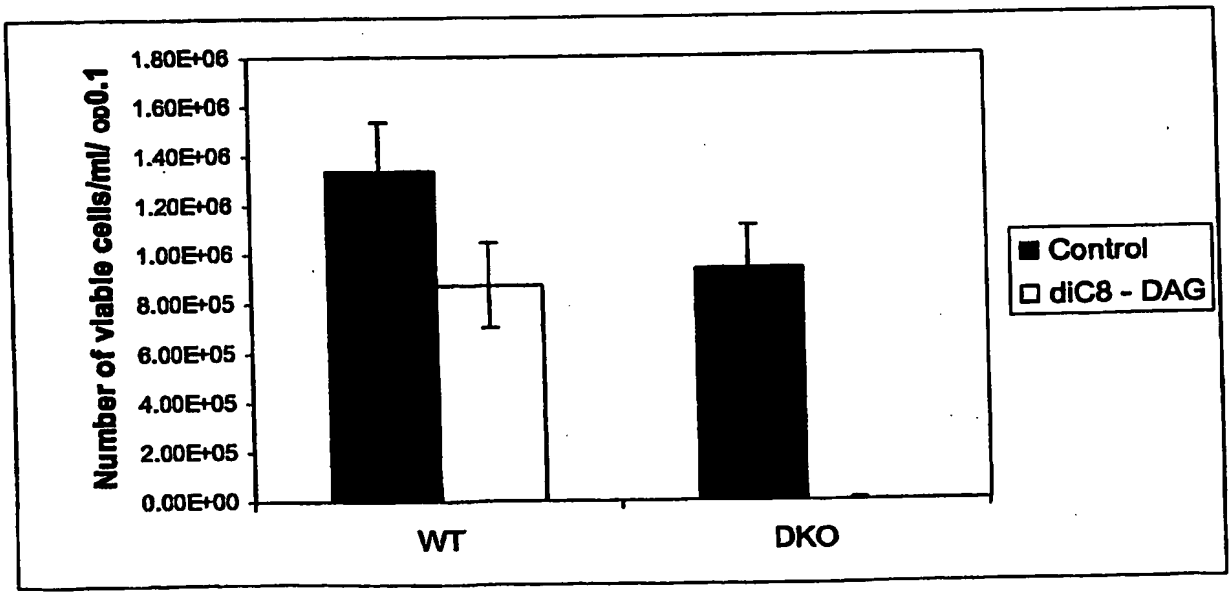
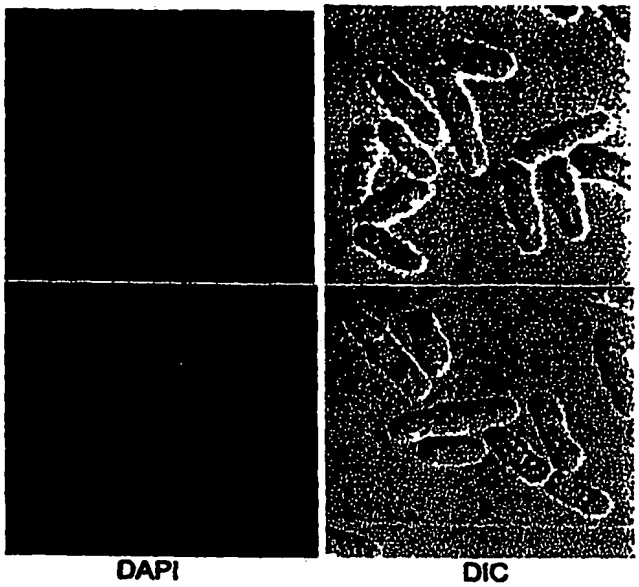


Zhang *et al.*, Figure 4 A, B

C



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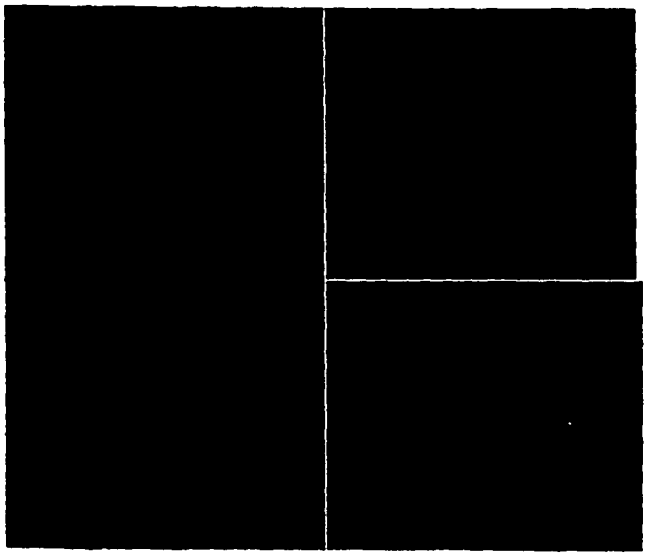
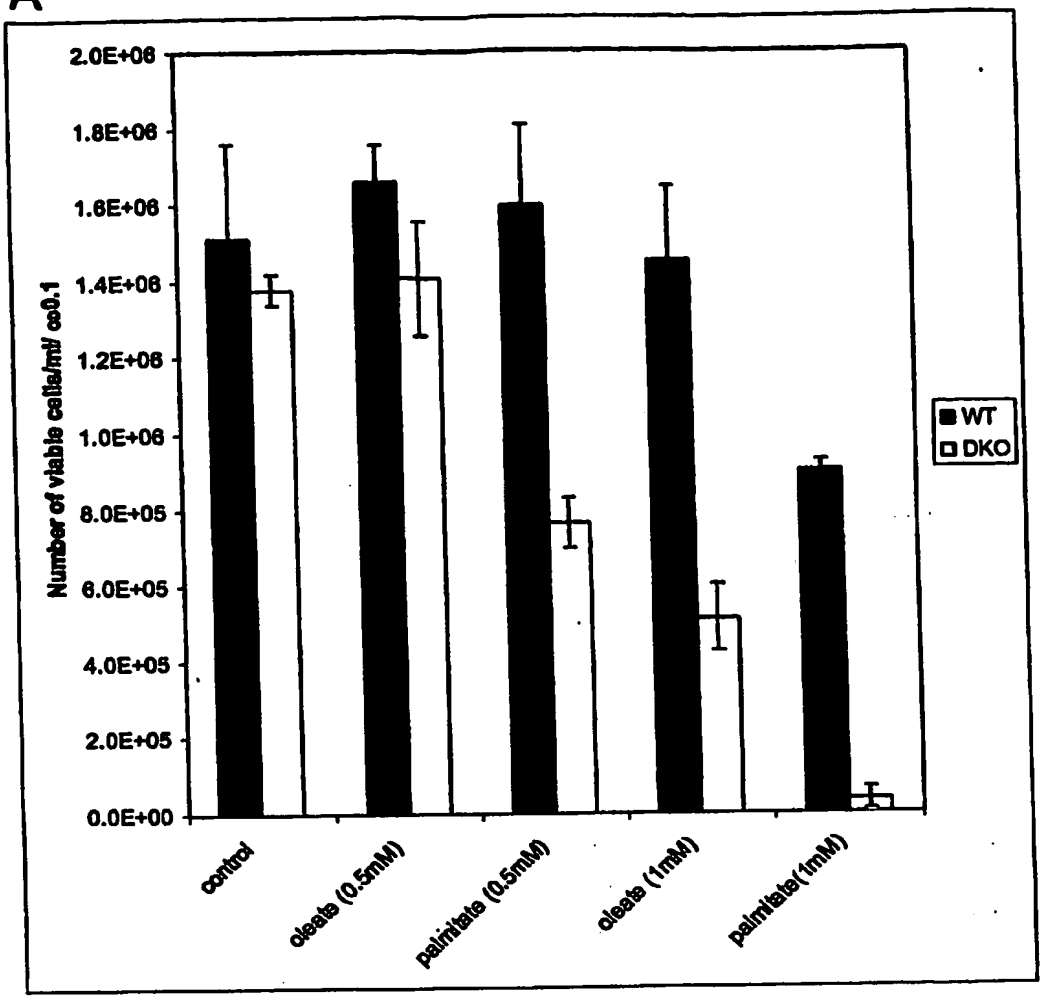
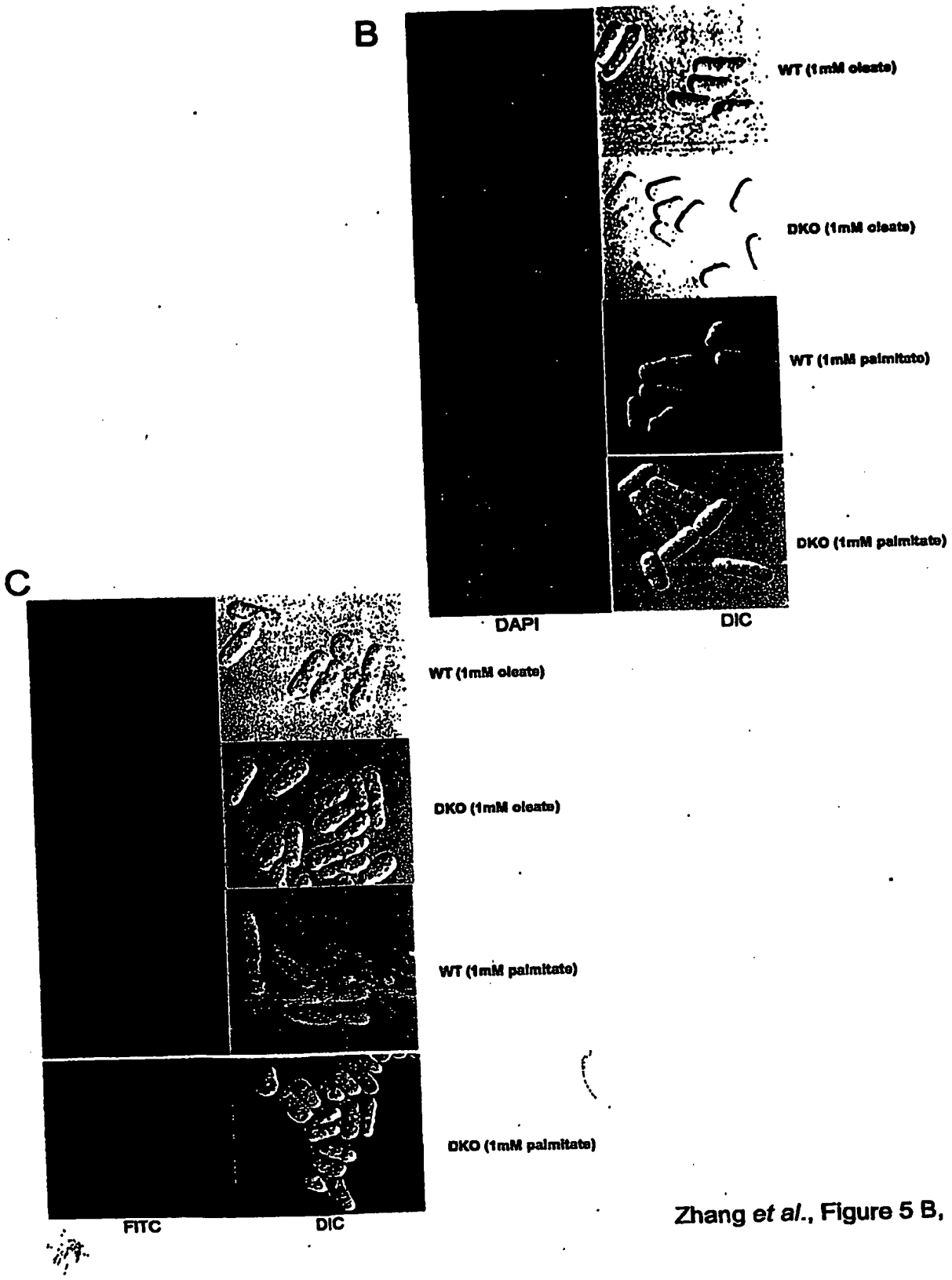


Zhang *et al.*, Figure 4 C, D

A

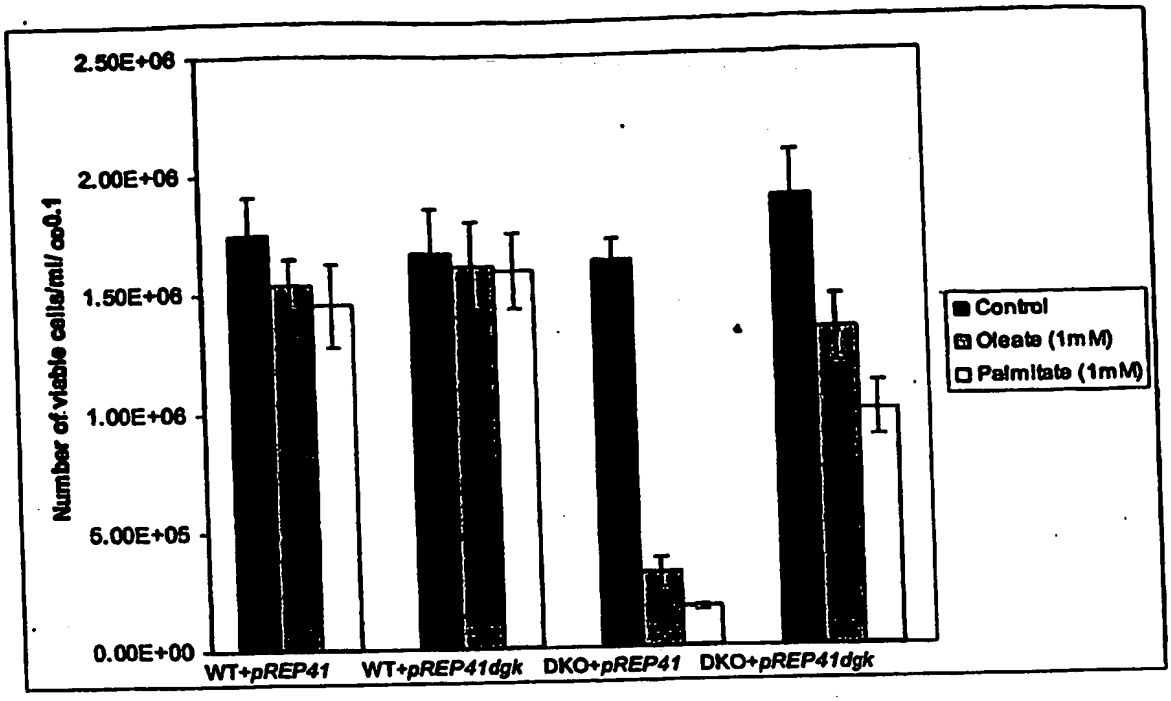


Zhang *et al.*, Figure 5 A

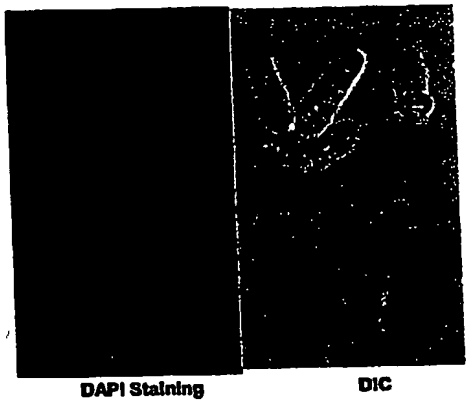


Zhang *et al.*, Figure 5 B, C

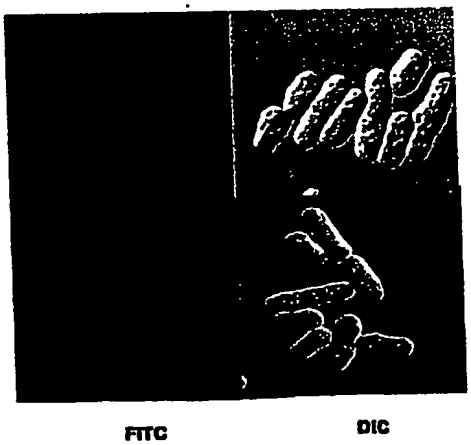
A



B

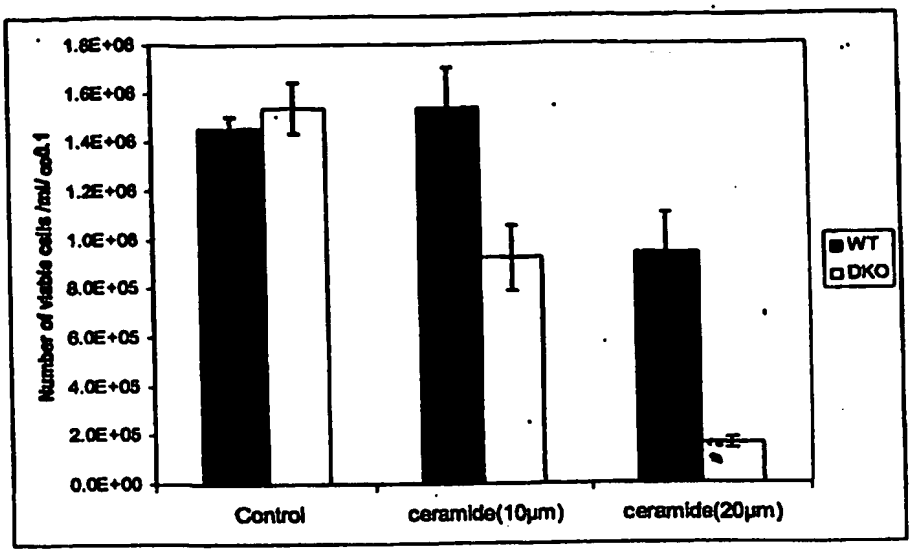


C



Zhang *et al.*, Figure 6 A, B, C

A



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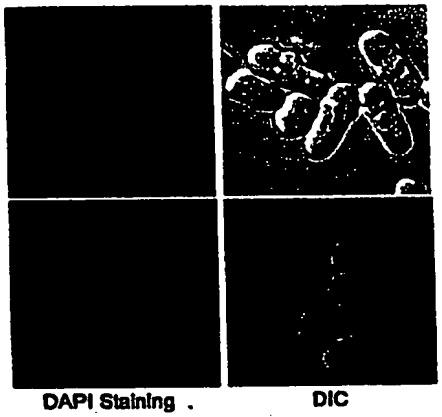
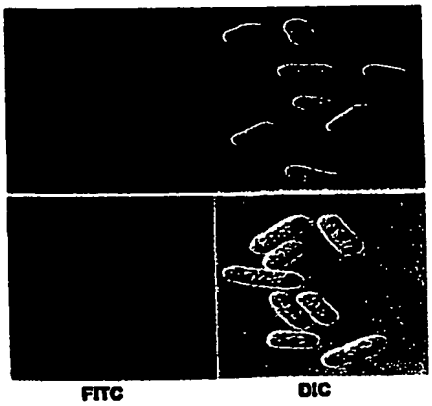
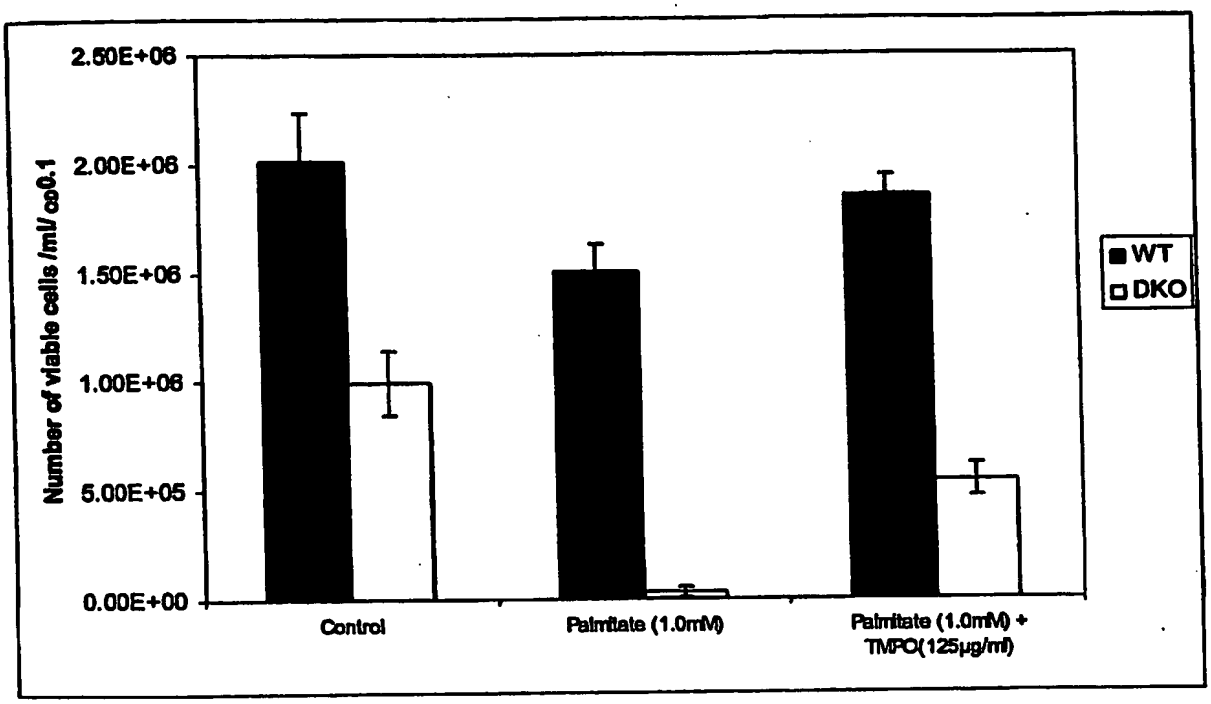


C



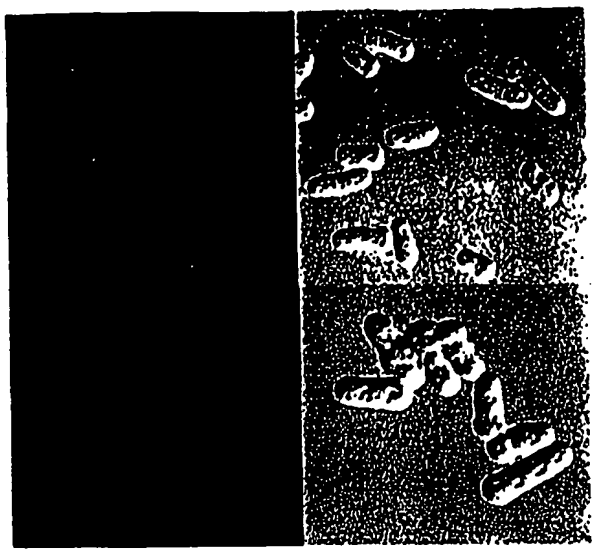
Zhang *et al.*, Figure 7 A, B, C

A



Zhang et al., Figure 8 A

B

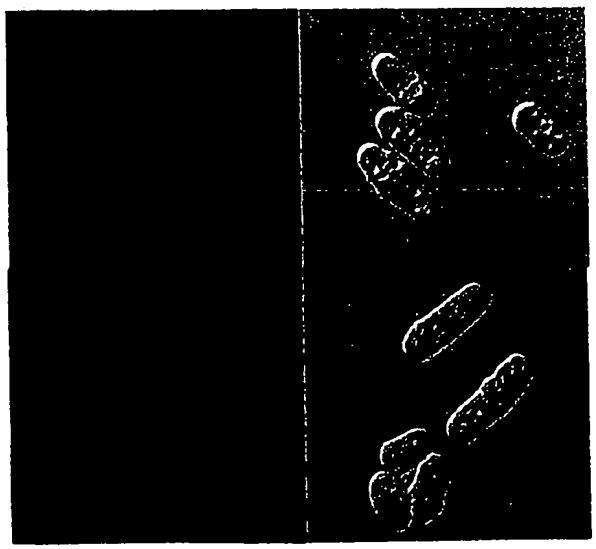


1 mM palmitate acid

1 mM palmitate +
125μg/ml TMPO

DAPI DIC

C



1 mM palmitate

1mM palmitate+
125μg/ml TMPO

Texas Red DIC

Zhang *et al.*, Figure 8 B, C

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